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An alternative origin for nanobacteria in kidney stones☆

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Abstract Small (50–200 nm), calcium phosphate (apatite)-covered organic particles called nanobacteria or calcifying nanoparticles (CNP) seem to be ubiquitous in kidney stones and are thought to be involved in stone formation. Although initial claims that these particles are the smallest known life forms have been somewhat softened, much controversy remains as to their involvement in kidney stone formation as well as in other pathological calcifications. I suggest that such particles are non-living and may be formed during the normal living activities of *bona-fide* bacteria which inhabit the kidneys. This hypothesis is based on previous observations that bacteria immersed in a supersaturated fluid produce organic globules which calcify when released to the surrounding fluid, forming CNP-like particles. The possibility that this process is responsible for the formation of CNP associated with pathological calcifications deserves greater scrutiny.

Introduction

Kidney stone formation (nephrolithiasis) is a common disease affecting all geographical, cultural and racial groups worldwide [1]. Kidney stones cause acute pain and necessitate various types of long-term medical management and surgical intervention. Furthermore, nephrolithiasis often indicates the existence of additional underlying disorders [1].

Kidney stones can be composed of a variety of minerals amalgamated with proteins [2]. Regardless of their mineralogy, two factors are fundamental in kidney stone development: supersaturation with respect to the forming mineral phase and crystal nucleation. Research has concentrated in understanding the metabolic and environmental factors which produce an abnormal urinary composition causing supersaturation [1,2]. Less is known about the controls on the nucleation of crystals which grow to form kidney stones.

Ten years ago, the claim that nanobacteria promote the nucleation of kidney stones provoked much controversy [3,4]. Nanobacteria are small (~50–200 nm diameter),

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calcium phosphate (apatite)-covered organic entities which have been proposed to be the smallest known living organisms [3,5]. Nanobacteria seem to occur in the majority of kidney stones [6] but have also been observed in other human and animal materials [7] as well as in sediments and rocks [8,9].

Based on the presence of apatite in the core of most kidney stones, on the widespread occurrence of nanobacteria in kidney stones and on the *in vitro* formation of kidney stone-like apatite in the presence of nanobacteria, nanobacteria have been indicated as the nucleating agents of kidney stones [3,6,10]. Traditionally, only struvite and some carbonate apatite stones are thought to be infectious in origin and are linked to alkalinity and phosphate production by urea-splitting bacteria [11]. According to Çiftçioglu et al. [6], instead, the formation of virtually all kidney stones is infectious because nanobacteria cause their nucleation.

At present, long-term medical treatment of kidney stones is directed at inhibiting the conditions that induce supersaturation in the urinary fluids, either by modifying dietary habits or through specific pharmacological treatment [1]. Understanding the process of kidney stone nucleation is important because if nucleation can be inhibited, additional means for treatment and prevention of kidney stones could be developed.

My hypothesis is that the nano-objects likely involved in the nucleation of kidney stones [3] are not living entities but are a by-product of the living activities of *bona-fide* bacteria inhabiting the kidney stones. If this hypothesis is confirmed, it could imply a direct involvement of *bona-fide* bacteria in the nucleation of kidney stones.

The nanobacteria controversy

The initial claim by Kajander and Çiftçiolu that nanobacteria are living organisms was based on nucleic acid stains, 16SrDNA sequencing, electron microscopy and the demonstration of a transferable biomineralization activity [3]. Following the protocols of Kajander and Çiftçioglu [3], Cisar et al. [11] succeeded in separating nanobacteria-like objects from fetal bovine serum. However, they showed that the putative nanobacterial 16SrDNA sequences could originate from contaminant organism. Furthermore, they showed that biomineralisation could be initiated on non-living macromolecules and transferred on ''subculture'' by self-propagating microcrystalline apatite. A more recent attempt by Drancourt et al. [13] failed to isolate nanobacteria in culture and to prove the bacterial nature of these nanoparticles in kidney stones. Miller et al. [14], on the other hand, report the successful culturing of nanobacteria-like structures isolated from human arteries and cardiac valves.

In the light of such mixed reports, the idea that nanobacteria are living organisms remains highly controversial, in part because of their very small size [15], and because their nucleic acid is not sequenced yet. Thus, the focus has momentarily moved on to the calcifying properties of nanobacteria, which are now called ''calcifying nanoparticles'' (CNP) [7], rather than on their living or non-living nature: what is the origin and composition of the internal, organic portion of CNP? How do CNP mineralize?

The role of bona-fide bacteria in the formation of CNP

In a microbial precipitation experiment designed to investigate the role of bacteria in the nucleation of calcium carbonate in geological environments, Aloisi et al. [16] describe a calcium carbonate nucleation process that offers a simple explanation for the formation of CNP and could be relevant to the nucleation of kidney stones. The experiment describes how a sulphate-reducing bacterium (*D. lacustre*), when immersed in a supersaturated culture medium, produces nanometer-sized organic globules which

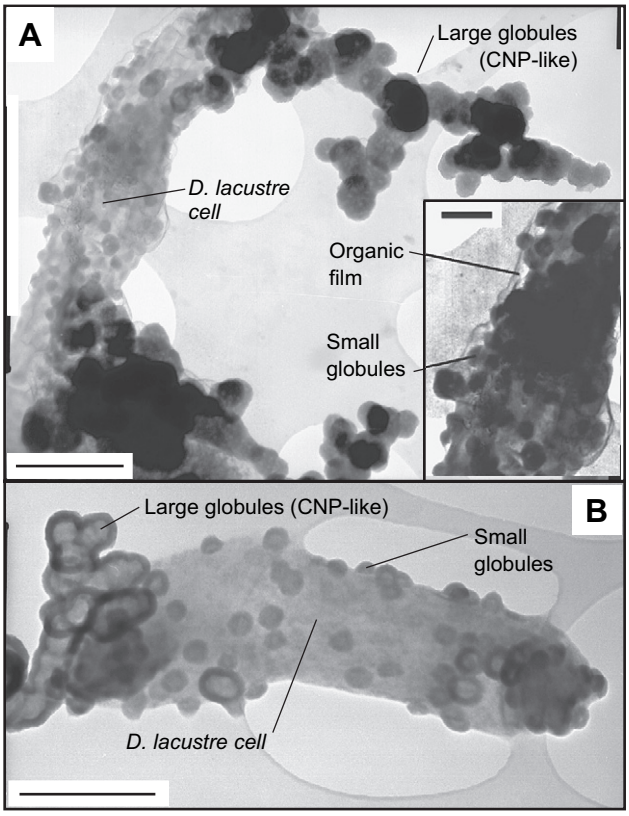

Figure 1 Transmission Electron Microscope images (modified from Aloisi et al. [16]) showing how the sulphate-reducing bacterium *D. lacustre* forms CNP-like particles when immersed in a supersaturated culture medium. (A) *D. lacustre* cell surrounded by small (60—110 nm), organic-rich globules intimately associated with the call surface. Large (110—200 nm), mineral-coated globules coalesce to form ''colonies'' in the surrounding fluid (scale bar 500 nm). Inset: small, organic-rich globules occur attached to the bacterial surface under a film of organic material probably composed of extracellular polymeric substances (scale bar 200 nm); (B) Detail of *D. lacustre* cell showing small, organic-rich globules on the cell surface and aggregates of large, mineral-coated globules attached to the polar end of the *D. lacustre* cell (scale bar 500 nm).

calcify significantly when they are released to the environmental fluid (Fig. 1; Ref. [16]).

The globules intimately associated with bacterial cells are consistently smaller (60—110 nm) and mineral-poor, compared to globules in the environmental fluids which are larger (110—200 nm) and mineralised. This is strong evidence for a bacterial origin of the organic nuclei and for a passive mechanism of mineralization on these nuclei once they are released to the supersaturated culture medium. The end product of this bacterial mineralization process shares many of the characteristics of CNP, including size (50—200 nm), an organic nucleus, a calcified mineral shell rich in phosphate (detected with Electron Energy Loss Spectroscopy) and the ability to form ''colonies'' (Fig. 1). Globule formation at the surface of bacterial cells does not occur in culture medium undersaturated with respect to calcium minerals or in control experiments which reproduce the chemistry (supersaturation) of the nucleation experiment but omit the sulphate-reducing bacteria.

In search for an alternative explanation for the origin of nanobacteria, other experiments have succeeded in producing nanobacteria-like objects via the nucleation of apatite on non-living macromolecules [12,17,18]. In these experiments, however, the nucleating organic macromolecules have been provided artificially, a situation highly unlikely to occur in the kidneys. The nucleation process described by Aloisi et al. [16], instead, provides an explanation for both the origin of the organic nuclei and for the formation of the mineral shell.

Kidney stones can harbour bacterial communities which, should supersaturation conditions arise, can potentially release organic globules which may serve as nucleation sites for various minerals. Sulphate-reducing bacteria are phylogenetically and metabolically distinct from the bacteria that can be present in kidneys [1]. However, the production of macromolecules such as exopolysaccharides and proteins and their release to the environment is a fundamental process involved in biofilm formation and is phylogenetically widespread amongst bacteria both in natural environments and in the human body [19]. It is macromolecules such as these that likely make up the inner part of the calcified globules observed by Aloisi et al. [16]. Indirect evidence that CNP found in kidneys could be produced by the process described by Aloisi et al. [16] comes from the claim that CNP isolated from kidney stones ''grow'' better in the presence of other bacteria [7].

Possible implications for other human pathological calcifications

Pathological calcification gives rise to a number of diseases and is increasingly attributed to bacterial infection. For example, CNP have been observed from calcified tissues including psammoma bodies of ovarian cancer [20] and human vascular tissue [14], and have been proposed as nucleating agents. Should the role of CNP in the formation of such pathological calcifications be confirmed, this would imply an even greater need to understand their nature and mechanism of formation. Such research efforts could benefit from ground truthing the hypothesis proposed in this article.

Testing the hypothesis

My hypothesis can be tested by observing the cell surfaces of bacteria that inhabit the kidneys when they are cultured in fluids supersaturated with respect to apatite. Two protocols should be followed: (1) pure cultures of urea-splitting bacteria, where supersaturation is induced by the metabolic activity of cultured organisms, and (2) pure cultures of other bacteria which are involved in urinary tract infections, where supersaturation is imposed by changing the chemistry of the experimental fluid. This second experiment simulates the response of bacteria in the kidneys to supersaturation produced by external factors (human metabolic or environmental). The bacterial surface should be observed regularly with TEM to check for the presence of calcified globules associated to the bacteria in supersaturated conditions.

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